

PA 1103516

RECEIVED

20 JAN 2004

WIPO

PCT

# THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

December 11, 2003

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: 60/434,264

FILING DATE: December 18, 2002

8E/03/1978

**PRIORITY  
DOCUMENT**

SUBMITTED OR TRANSMITTED IN  
COMPLIANCE WITH RULE 17.1(a) OR (b)



By Authority of the  
COMMISSIONER OF PATENTS AND TRADEMARKS

*P. R. Grant*

P. R. GRANT

Certifying Officer

BEST AVAILABLE COPY

12/18/02  
1c960 U.S. PTO

12-19-02 60434264 12/18/02

Please type a plus sign (+) inside this box ☒

Approved for use through 10/31/2002. OMB 0651-0032  
U.S. Patent and Trademark Office, U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

## PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No.

EF 253633433 US

INVENTOR(s)				
Given Name (first and middle (if any))	Family Name or Surname	Residence (City and either State or Foreign Country)		
Jay Jie	Liu	Wilmington, Delaware		
<input checked="" type="checkbox"/> Additional inventors are being named on the <u>1</u> separately numbered sheets attached hereto				
TITLE OF THE INVENTION (280 characters max)				
INOSITOL PHOSPHATE DETECTION ASSAYS				
Direct all correspondence to: CORRESPONDENCE ADDRESS				
<input checked="" type="checkbox"/> Customer Number		22466		Place Customer Number Bar Code Label here
OR		Type Customer Number here		
<input type="checkbox"/> Firm or Individual Name				
Address				
Address				
City		State	ZIP	
Country		Telephone	Fax	
ENCLOSED APPLICATION PARTS (check all that apply)				
<input checked="" type="checkbox"/> Specification	Number of Pages	23	<input type="checkbox"/> CD(s), Number	
<input checked="" type="checkbox"/> Drawing(s)	Number of Sheets	7	<input type="checkbox"/> Other (specify)	
<input type="checkbox"/> Application Data Sheet. See 37 CFR 1.76				
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT				
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27.			FILING FEE AMOUNT (\$)	
<input type="checkbox"/> A check or money order is enclosed to cover the filing fees				
<input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number:			26-0166	
<input type="checkbox"/> Payment by credit card. Form PTO-2038 is attached.			\$160.00	
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.				
<input checked="" type="checkbox"/> No.				
<input type="checkbox"/> Yes, the name of the U.S. Government agency and the Government contract number are: _____				

Respectfully submitted,

SIGNATURE

TYPED or PRINTED NAME

TELEPHONE

Robin S. Quartin, Ph.D.

(302) 885-9129

Date 12 / 18 / 02

REGISTRATION NO.  
(if appropriate)  
Docket Number:

45,028

100933-1 US

## USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

**PROVISIONAL APPLICATION COVER SHEET**  
*Additional Page*

PTO/SB/16 (02-01)

Approved for use through 10/31/2002. OMB 0651-0032  
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.		Docket Number	100933-1 US	Type a plus sign (+) inside this box →	+
INVENTOR(S)/APPLICANT(S)					
Given Name (first and middle [if any])		Family or Surname	Residence (City and either State or Foreign Country)		
Deborah S.		Hartman	Wilmington, Delaware		
James Robert		Bostwick	Wilmington, Delaware		

Number 2 of 2

**WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.**

60434264 - 121802

PTO/SB/17 (10-01)

Approved for use through 10/31/2002. OMB 0651-0032  
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

**FEE TRANSMITTAL  
for FY 2002**

Patent fees are subject to annual revision.

**TOTAL AMOUNT OF PAYMENT** (\$) 160.00**Complete if Known**

Application Number	not yet assigned
Filing Date	December 18, 2002
First Named Inventor	Liu et al.
Examiner Name	not yet assigned
Group Art Unit	not yet assigned
Attorney Docket No.	100933-1 US

**METHOD OF PAYMENT**

- 1.
- ☒
- The Commissioner is hereby authorized to charge indicated fees and credit any overpayments to:

Deposit Account Number	26-0166
Deposit Account Name	

☐ Charge Any Additional Fee Required Under 37 CFR 1.16 and 1.17☐ Applicant claims small entity status. See 37 CFR 1.27

- 2.
- ☐
- Payment Enclosed:

☐ Check ☐ Credit card ☐ Money Order ☐ Other**FEE CALCULATION****1. BASIC FILING FEE**

Large Entity Fee Code (\$)	Small Entity Fee Code (\$)	Fee Description	Fee Paid
101 740	201 370	Utility filing fee	
106 330	206 165	Design filing fee	
107 510	207 255	Plant filing fee	
108 740	208 370	Reissue filing fee	
114 160	214 80	Provisional filing fee	160.00

**SUBTOTAL (1)** (\$) 160.00**2. EXTRA CLAIM FEES**

Total Claims	Extra Claims	Fee from below	Fee Paid
Independent Claims	-20** =	X	
Multiple Dependent Claims	-3** =	X	

Large Entity Fee Code (\$)	Small Entity Fee Code (\$)	Fee Description
103 18	203 9	Claims in excess of 20
102 84	202 42	Independent claims in excess of 3
104 280	204 140	Multiple dependent claim, if not paid
109 84	209 42	** Reissue independent claims over original patent
110 18	210 9	** Reissue claims in excess of 20 and over original patent

**SUBTOTAL (2)**

(\$)

\*\*or number previously paid, if greater; For Reissues, see above

**FEE CALCULATION (continued)****3. ADDITIONAL FEES**

Large Entity Fee Code (\$)	Small Entity Fee Code (\$)	Fee Description	Fee Paid
105 130	205 65	Surcharge - late filing fee or oath	
127 50	227 25	Surcharge - late provisional filing fee or cover sheet	
139 130	139 130	Non-English specification	
147 2,520	147 2,520	For filing a request for <i>ex parte</i> reexamination	
112 920*	112 920*	Requesting publication of SIR prior to Examiner action	
113 1,840*	113 1,840*	Requesting publication of SIR after Examiner action	
115 110	215 55	Extension for reply within first month	
116 400	216 200	Extension for reply within second month	
117 920	217 460	Extension for reply within third month	
118 1,440	218 720	Extension for reply within fourth month	
128 1,960	228 980	Extension for reply within fifth month	
119 320	219 160	Notice of Appeal	
120 320	220 160	Filing a brief in support of an appeal	
121 280	221 140	Request for oral hearing	
138 1,510	138 1,510	Petition to institute a public use proceeding	
140 110	240 55	Petition to revive - unavoidable	
141 1,280	241 640	Petition to revive - unintentional	
142 1,280	242 640	Utility issue fee (or reissue)	
143 460	243 230	Design issue fee	
144 620	244 310	Plant issue fee	
122 130	122 130	Petitions to the Commissioner	
123 50	123 50	Processing fee under 37 CFR 1.17(q)	
126 180	126 180	Submission of Information Disclosure Stmt	
581 40	581 40	Recording each patent assignment per property (times number of properties)	
146 740	246 370	Filing a submission after final rejection (37 CFR § 1.129(a))	
149 740	249 370	For each additional invention to be examined (37 CFR § 1.129(b))	
179 740	279 370	Request for Continued Examination (RCE)	
169 900	169 900	Request for expedited examination of a design application	

Other fee (specify)

\*Reduced by Basic Filing Fee Paid

**SUBTOTAL (3)** (\$)**SUBMITTED BY**

Name (Print/Type) Robin S. Quartin, Ph.D.

Registration No. 45,028  
(Attorney/Agent)**Complete (if applicable)**

Telephone 302-885-9129

Signature

Date 12/18/2002

**WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.**

Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.

## INOSITOL PHOSPHATE DETECTION ASSAYS

### FIELD OF THE INVENTION

[0001] The present invention relates to methods of detecting inositol phosphate, assays for measuring the activity of phospholipase C-linked receptors, kinases, and phosphatases, and assays for screening for compounds that modulate the activity of these receptors and enzymes.

### BACKGROUND

[0002] The regulation of phosphoinositide metabolism is a key component in cell signaling networks (Michell, 1992, Trends Biochem. Sci., 17:274-276; Payraastre *et al.*, 2001, Cell Signal, 13:377-387). A great number of hormones, growth factors, cytokines and neurotransmitters communicate with cell interior via receptors coupled to phospholipase C (PLC). Activation of PLC- $\beta$  through the heterotrimeric G protein pathway or PLC- $\gamma$  through the tyrosine kinase pathway leads to an increase in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) and the generation of two ubiquitous second messengers, inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol, which then trigger the subsequent signaling cascades and cellular events (Martin, 1991, Pharmacol. Ther., 49:329-345). While this system has been under intensive studies for decades, no significant improvement has been made in the methodology of phosphoinositide hydrolysis measurement. Currently, the most widely used method was developed 20 years ago by Berridge which employs solvent extraction and anion-exchange chromatography to separate labeled inositol phosphate from inositol and phosphoinositide lipid after receptor stimulation in the presence of LiCl (Berridge *et al.*, 1982, Biochem. J., 206(3):587-595). Although highly sensitive, this method suffers from significant limitations, such as the time-consuming and labor-intensive processing of samples, which results in low throughput and the generation of a large volume of radioactive waste.

[0003] A number of PLC-linked receptors are molecular targets for therapeutic interventions. Since the increase in phosphoinositide hydrolysis is directly linked to receptor activation, its

measurement has been frequently used as a functional assay to study the interactions of pharmacological agents with their receptors. In addition, the measurement of phosphoinositide (PI) hydrolysis can be used to identify novel agonists, antagonists, or modulators acting at receptors that are coupled to PLC activation. Recent advances in generating a large number of compounds through modern medicinal chemistry technologies together with ever increasing number of novel molecular targets identified from genomic efforts have increased the pressure to develop methodologies to enable rapid evaluation of compound libraries to identify lead chemical structures.

[0004] Inositol monophosphatase (IMPase), the enzyme that dephosphorylates inositol monophosphates to regenerate inositol, is associated with mental disease. Specifically, IMPase activity in cerebrospinal fluid (CSF) is significantly increased in patients suffering from depression, bipolar disease, and schizophrenia, and lithium treatment can return IMPase activity to normal levels in bipolar patients (Atack, 1996, Brain Res. Rev., 22:183-190; Atack et al., 1998, Biol. Psychiatry, 44:433-437). Compounds that can inhibit IMPase are useful in the treatment of bipolar disorders. In addition, CSF IMPase activity serves as a marker for mental illness.

[0005] Inositol-1-phosphate synthase (INPS) catalyses the addition of phosphate to inositol, and is responsible for the production of inositol phosphate in archaea and eubacteria (Bachhawat *et al.*, 2002, Trends Genet., 16:111-113). INPS is a key enzyme involved in the phosphatidylinositol (PI) biosynthetic pathway (Norman *et al.*, 2002, Structure, 10:393-402). The enzyme is known to be overexpressed in isoniazid resistant strains of *Mycobacterium tuberculosis*, and has been shown to be important for its virulence. Since PI is essential for *M. tuberculosis* viability, INPS is a target for antimycobacterial agents and drugs.

[0006] Ideally, receptor-stimulated PI hydrolysis would be monitored as the rate of production of IP<sub>3</sub> in the absence of degradation, *i.e.*, in a manner analogous to the measurement of cAMP accumulation in the presence of phosphodiesterase inhibitors. However, the metabolism of IP<sub>3</sub> occurs rapidly in most cells and specific cell-permeant inhibitors of enzymes that metabolize IP<sub>3</sub> have yet to be identified (Fisher, 1995, Eur. J. Pharmacol., 288:231-250; Wojcikiewicz *et al.*, 1993, Trends Pharmacol. Sci., 14:279-285). As an alternative, measurement of PI hydrolysis has been performed in the presence of LiCl, which blocks inositol monophosphatase and results in the accumulation of IP<sub>3</sub> metabolites, most notably inositol-1-phosphate (IP<sub>1</sub>). The assay has been commonly performed on cells pre-incubated with [<sup>3</sup>H]inositol. In these cells, phosphoinositides, including phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-

bisphosphate, become radiolabeled and hydrolysis of these lipids by PLC generates [ $^3\text{H}$ ]inositol phosphates, which are isolated by ion exchange chromatography and quantified by liquid scintillation counting (Berridge *et al.*, 1982, *supra*; Berridge *et al.*, 1984, *Biochem. J.*, 222:195-201; Liu *et al.*, 1996, *J. Biol. Chem.*, 271:6172-6178).

[0007] The principle of Immobilized Metal Affinity Chromatography (IMAC) makes use of matrix-bound metals to purify biomaterials on the basis of their interaction with the immobilized metal ions (Porath *et al.*, 1983, *Biochemistry*, 22:1621-1630; Sulkowski, 1989, *Bioessays*, 10:170-175; Yip *et al.*, 1996, *Methods Mol. Biol.*, 59:197-210; Gaberc-Porekar *et al.*, 2001, *J. Biochem. Biophys. Methods*, 49:335-360). It has been used for about two decades to purify proteins, peptides and nucleic acids (Sulkowski, 1985, *Trends Biotechnol.*, 3:1-7; Jiang *et al.*, 1996, *Anal. Biochem.*, 242:45-54; Haupt *et al.*, 1996, *Anal. Biochem.*, 234:149-154). The most common application is using  $\text{Ni}^{2+}$ -column to purify polyhistidine-tagged proteins. Based on the original observation by Anderson and Porath that immobilized  $\text{Fe}^{3+}$  bound free phosphate with high affinity,  $\text{Fe}^{3+}$ -IMAC has been successfully used to isolate phosphorylated peptides (Andersson *et al.*, 1986, *Anal. Biochem.*, 154:250-254; Li S, *et al.*, 1999, *Anal. Biochem.*, 270:9-14; Scanff *et al.*, 1991, *J. Chromatogr.*, 539:425-432). The strong interaction of the phosphate group with  $\text{IDA-Fe}^{3+}$  is believed to be due to the formation of two coordination bonds resulting in a four-member ring complex (Andersson *et al.*, *supra*; Chaga *et al.*, 1992, *J. Chromatogr.*, 627:163-172; Muszynska *et al.*, 1986, *Biochemistry*, 25:6850-6853; Holmes *et al.*, 1997, *J. Liq. Chromatogr. Rel. Technol.*, 20:123-142).

Historically, several approaches have been used to study PI hydrolysis, but are now rarely used, which include the measurement of the increased  $^{32}\text{P}$ -labelling of either phosphatidate or PI as a secondary event of PI hydrolysis and the measurement of  $\text{IP}_3$  by mass spectroscopy following HPLC (Fisher, *supra*; Wojcikiewicz *et al.*, *supra*; Shears, 1992, in *Inositol Phosphates and Calcium Signalling (Advances in Second Messenger and Phosphoprotein Research)*, Vol 26, pp 63 – 92, J.W. Putney, Jr. (ed.), Raven Press, New York). Recently, a ligand binding assay has been reported for the measurement of  $\text{IP}_3$  as an index of PI hydrolysis (Viko *et al.*, 1998, *Pharmacol Toxicol*, 83:23-28; Hanem *et al.*, 1996, *Mol. Cell. Biochem.*, 164:167-172), which employs specific receptors or binding proteins purified from adrenal gland or other tissues to capture 1,4,5- $\text{IP}_3$  exclusively (Guillemette *et al.*, 1987, *Proc. Natl. Acad. Sci. U.S.A.*, 84:8195-9199; Baukal *et al.*, 1985, *Biochem. Biophys. Res. Commun.*, 133:532-538; Theibert *et al.*, 1987, *Biochem. Biophys. Res. Commun.*, 148:1283-9). Amersham Biosciences Corp. (Piscataway, NJ) and PerkinElmer (Boston, MA) have both

developed a RIA kit (TRK1000 and NEK064, respectively) for this assay technique. Despite the advantages of measuring the most relevant species of inositol phosphates, the use of this method is limited by the fact that  $IP_3$  is rapidly metabolized in the cell resulting in a transient elevation of  $IP_3$  with a half-life less than a minute (Hughes *et al.*, 1989, J. Biol. Chem., 264:9400-9407; Fisher *et al.*, 1992, J. Neurochem., 58:18-38; Fisher *et al.*, 1990, Mol. Pharmacol., 38:54-63). This requires precise control or timing of the agonist stimulation and the termination of the reaction in the scale of seconds. In addition, the  $IP_3$  peak time is variable according to the temperature, pH, the association rate and the concentration of the agonist compound. The success of this method will depend on the future identification of cell-permeant inhibitors of the  $IP_3$ -metabolizing enzymes, 5-phosphatase and  $IP_3$  3-kinase.

#### SUMMARY

[0008] The present invention provides methods for detecting phospholipase C-linked receptor activation comprising, providing cells expressing a receptor that utilizes a phospholipase C signaling pathway, contacting the cells with labeled inositol, contacting the cells with a receptor agonist, whereby labeled inositol phosphate is generated, releasing the labeled inositol phosphate from the cells, contacting the cells with an immobilized metal ion under conditions permitting inositol phosphate to bind to the metal ion; and detecting labeled inositol phosphate bound to the immobilized metal ion, wherein bound labeled inositol phosphate is indicative of receptor activation.

[0009] The present invention also provides methods for identifying compounds that modulate phospholipase C-linked receptor activation comprising, in the presence or absence of a compound, providing cells expressing a receptor that utilizes a phospholipase C signaling pathway, contacting the cells with labeled inositol, contacting the cells with a receptor agonist, whereby labeled inositol phosphate is generated, releasing labeled inositol phosphate from the cells, contacting the labeled inositol phosphate with an immobilized metal ion under conditions to allow inositol phosphate to bind to the metal ion, detecting labeled inositol phosphate bound to the immobilized metal ion, wherein an alteration in the amount of bound labeled inositol phosphate in the presence of a compound identifies said compound as a compound that modulates phospholipase C-linked receptor activation.

[0010] The present invention also provides methods for detecting inositol monophosphatase activity in a sample comprising contacting the sample with labeled inositol phosphate under conditions permitting inositol monophosphatase to hydrolyze phosphate from inositol phosphate, contacting the sample with an immobilized metal ion under conditions permitting



inositol phosphate to bind to the metal ion, and detecting labeled inositol phosphate bound to the immobilized metal ion, wherein a decrease in the amount of bound labeled inositol phosphate is indicative of inositol monophosphatase activity in the sample.

[0011] The present invention also provides methods for identifying compounds that modulate inositol monophosphatase activity comprising, in the presence or absence of a compound, contacting inositol monophosphatase with labeled inositol phosphate under conditions permitting inositol monophosphatase to hydrolyze phosphate from inositol phosphate, contacting the reaction mixture with an immobilized metal ion under conditions permitting inositol phosphate to bind to the metal ion, and detecting labeled inositol phosphate bound to the immobilized metal ion, wherein an alteration in the amount of bound labeled inositol phosphate in the presence of a compound identifies said compound as a compound that modulates inositol monophosphatase activity.

[0012] The present invention also provides methods for detecting inositol-1-phosphate synthase activity in a sample comprising contacting the sample with labeled inositol under conditions permitting inositol-1-phosphate synthase to catalyze the addition of phosphate to inositol, contacting the sample with an immobilized metal ion under conditions permitting inositol phosphate to bind to the metal ion, and detecting labeled inositol phosphate bound to the immobilized metal ion, wherein an increase in the amount of bound labeled inositol phosphate is indicative of inositol-1-phosphate synthase activity in the sample.

[0013] The present invention also provides methods for identifying compounds that modulate inositol-1-phosphate synthase activity comprising, in the presence or absence of a compound, contacting inositol-1-phosphate synthase with labeled inositol under conditions permitting inositol-1-phosphate synthase to catalyze the addition of phosphate to inositol, contacting the reaction mixture with an immobilized metal ion under conditions permitting inositol phosphate to bind to the metal ion, and detecting labeled inositol phosphate bound to the immobilized metal ion, wherein an alteration in the amount of bound labeled inositol phosphate in the presence of a compound identifies said compound as a compound that modulates inositol-1-phosphate synthase activity.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[0014] Figures 1A and 1B show binding of [ $^3\text{H}$ ]inositol and [ $^3\text{H}$ ]inositol-1-phosphate to the SPA beads loaded with different metal ions. 10 nCi of [ $^3\text{H}$ ]inositol or [ $^3\text{H}$ ]inositol-1-phosphate were incubated with 1 mg metal ion-loaded SPA beads. After vacuum filtration, the radioactivity that remained in the flow-through samples was measured by liquid

scintillation counting (Figure 1A), and the radioactivity retained on the beads was detected by SPA technology (Figure 1B). Each data point represents the mean  $\pm$ SD of triplicate samples.

[0015] Figure 2 shows the pH-dependent binding of 10 nCi [ $^3$ H]inositol-1-phosphate to 1 mg  $Zr^{4+}$ -loaded SPA beads. Each data point represents the mean  $\pm$  SD of triplicate samples.

[0016] Figure 3 shows the blockade of the binding of 10 nCi [ $^3$ H]inositol-1-phosphate to 2 mg  $Zr^{4+}$ -loaded SPA beads by pretreatment of the beads with unlabeled inositol-1-phosphate or ATP. Each data point represents the mean  $\pm$ SD of triplicate samples.

[0017] Figure 4 shows the measurement of NK1-mediated PI hydrolysis in the cells stimulated with 0.1  $\mu$ M Substance P or saline by using 2 mg SPA beads loaded with different metal ions. Each data point represents the mean  $\pm$ SD of triplicate samples.

[0018] Figure 5 shows the titration of the amount of  $Zr^{4+}$ -SPA beads for the measurement of 0.1  $\mu$ M Substance P-stimulated PI hydrolysis in 96-well plates. Each data point represents the mean  $\pm$ SD of triplicate samples.

[0019] Figure 6 shows the concentration-dependent stimulation of NK1-mediated PI hydrolysis by Substance P with or without the pretreatment of the cells with NK1 antagonist L-733060 at 10, 50 and 250 nM. The responses were measured with 2 mg/well  $Zr^{4+}$ -SPA beads. Each data point represents the mean  $\pm$ SD of duplicate samples.

[0020] Figure 7 shows the concentration-dependent stimulation of PI hydrolysis by PDGF-BB in quiescent Swiss 3T3 cells. The responses were measured by using 2 mg/well  $Zr^{4+}$ -SPA beads. Each data point represents the mean  $\pm$ SD of duplicate samples.

#### DETAILED DESCRIPTION

[0021] The present invention is based upon our discovery that immobilized metal ions can be used as affinity ligands to entrap inositol phosphates. Since immobilized metal ions bind inositol phosphate, rather than inositol, inositol phosphates generated from a variety of enzymatic and biochemical reactions can be measured without any separation steps and

without the use of scintillation cocktails. The methods of the present invention are broadly applicable in signal transduction research and drug discovery.

[0022] Our discovery can be harnessed in methods for detecting the presence of inositol phosphate in a sample. These assays can be used to quantitate inositol phosphate for monitoring its formation and degradation, for monitoring the activity of enzymes and/or pathways involved in inositol phosphate metabolism, and for screening for compounds that modulate the activities of such enzymes and/or pathways. The detection methods of the present invention are based upon using immobilized metal ions to bind inositol phosphate, such that the bound inositol phosphate can then be measured or quantitated.

[0023] The present invention provides methods for detecting inositol phosphate in a sample, where the methods comprise contacting the sample with an immobilized metal ion under conditions permitting inositol phosphate to bind to the metal ion, and measuring inositol phosphate bound to the immobilized metal ion, wherein inositol phosphate bound to the immobilized metal ion is indicative of inositol phosphate in the sample.

[0024] The sample that is analyzed can be from any source, including, but not limited to biological, medical, and *in-vivo* or *in vitro* reaction mixtures. The methods of the present invention can therefore be used to monitor the activity of enzymes, proteins or systems that generate or degrade inositol phosphate. Any method of measuring bound inositol phosphate can be used. For example, the bound inositol phosphate can be measured on the basis of an incorporated detectable label.

[0025] Our discovery can be harnessed in assays designed to monitor receptor-mediated phosphoinositide hydrolysis and in assays designed to monitor or detect the activity of enzymes that phosphorylate inositol or enzymes that hydrolyse inositol phosphate. The present invention provides assays for measuring the activation and activity of a variety of phospholipase C-linked receptors. We have used IMAC-SPA beads to measure the inositol phosphate responses mediated by the G protein-coupled neurokinin NK1 receptor and the platelet-derived growth factor (PDGF) cytokine receptor. The present invention also provides assays for detecting the presence of and measuring the activity of inositol monophosphatase and inositol-1-phosphate synthase. The present invention also provides assays for identifying compounds that modulate the activities of such receptors and enzymes.

[0026] As used herein, the terms "modulate" or "modulates" in reference to receptor activation or enzymatic activity include any measurable alteration to receptor activation or enzymatic activity. Modulation of receptor activation includes activation, inhibition and potentiation of the activation by an agonist (natural or otherwise) of the receptor.

Compounds that modulate receptor activity include agonists, antagonists and inverse agonists of the receptor. Modulation of the activity of an enzyme includes, but is not limited to, activation, enhancement, and inhibition of enzymatic activity.

[0027] As used herein, the terms "contact" or "contacting" refers to any method of combining and bringing into contact various components such as test compounds, cells, labeled inositol, labeled inositol phosphate, enzymes, or receptor agonists. For example, components can be brought into contact with cells by adding the components to the culture medium in a wide variety of culture vessels, tubes, plates, etc. Components can also be brought into contact in cell-free reaction solutions in a wide variety of reaction vessels, tubes, plates, etc.

[0028] As used herein, the term "increase" in reference to amount of metal ion-bound labeled inositol phosphate refers to any measurable augmentation of the amount of bound labeled inositol phosphate.

[0029] As used herein, the term "decrease" in reference to amount of metal ion-bound labeled inositol phosphate refers to any measurable diminution of the amount of bound labeled inositol phosphate.

[0030] One aspect of the present invention is directed to methods of detecting the activation of phospholipase C-linked receptors in cells expressing a receptor that utilizes a phospholipase C signaling pathway comprising contacting the cells with labeled inositol, contacting the cells with a receptor agonist, whereby labeled inositol phosphate is generated, releasing the labeled inositol phosphate from the cells, contacting the cells with an immobilized metal ion under conditions permitting inositol phosphate to bind to the metal ion; and detecting labeled inositol phosphate bound to the immobilized metal ion, wherein bound labeled inositol phosphate is indicative of receptor activation.

[0031] Any cells in which a phospholipase C-linked receptor is expressed or can be engineered to be expressed can be used. Such cells include, but are not limited to, mammalian cells including, but not limited to, human, hamster, mouse, rat, or monkey, and non-mammalian cells such as amphibian (*e.g.*, frog), fish cells, insect cells, and yeast cells.

[0032] Any receptor that causes formation of inositol phosphate as a result of its activation can be assayed in the methods of the present invention. By way of non-limiting example, the methods of the present invention can be used to assay membrane-linked receptors that are linked to phospholipase C activation, including, but not limited to, members of the seven transmembrane domain G protein-coupled receptor superfamily, *e.g.*, neurokinin NK1 receptor and muscarinic m1 acetylcholine receptor, and members of the single

transmembrane domain tyrosine kinase-linked receptor superfamily, *e.g.*, PDGF receptor and NGF receptor.

[0033] In some embodiments of the present invention the receptor is a seven transmembrane domain G protein-coupled receptor or a single transmembrane domain tyrosine kinase-linked receptor. In some embodiments of the present invention, the receptor is selected from neurokinin NK1 receptor, muscarinic m1 acetylcholine receptor, PDGF receptor, and NGF receptor.

[0034] A receptor agonist is any ligand that activates the receptor of interest. There are many known ligand-agonist/receptor pairs. By way of non-limiting example, Substance P is an agonist of the neurokinin NK1 receptor, and platelet-derived growth factor (PDGF) is an agonist of the PDGF receptor.

[0035] Any traceable, detectable, or measurable label may be used for labeling the inositol used in the methods of the present invention. The labels that can be used to label the inositol include, but are not limited to, radiolabels, fluorescent labels, chemiluminescent labels, and haptens (*e.g.*, biotin, digoxin).

[0036] In some embodiments of the present invention, the inositol label is selected from a radiolabel, a fluorescent label, a chemiluminescent label, or a hapten.

[0037] In some embodiments of the present invention, the inositol label is a radiolabel.

[0038] Releasing the inositol phosphate (including labeled inositol phosphate) from cells can be achieved by many methods known to those of skill in the art, including, but not limited to, mixing or treating the cells with a hypotonic solution or a detergent, or sonication.

[0039] Any metal ion that will bind inositol phosphate can be used in the methods of the present invention. By way of non-limiting example, metal ions that can be used with the present invention include  $Zr^{4+}$ ,  $Ga^{3+}$ ,  $Al^{3+}$ ,  $Fe^{3+}$ ,  $Sc^{3+}$ , and  $Lu^{3+}$ . Conditions permitting inositol phosphate to bind the aforementioned metal ions include a pH in the range of from about 2.0 to about 6.0.

[0040] In some embodiments of the present invention, the metal ion is selected from  $Zr^{4+}$ ,  $Ga^{3+}$ ,  $Al^{3+}$ ,  $Fe^{3+}$ ,  $Sc^{3+}$ , and  $Lu^{3+}$ . In some embodiments of the present invention the metal ion is  $Zr^{4+}$ .

Metal ions can be immobilized by affixing or otherwise attaching them to a solid support. For example, metal ions can be immobilized to an affinity matrix, which is a solid support having metal ion-chelating compounds covalently attached to it. Metal ion-chelating compounds include, but are not limited to, iminodiacetic acid (IDA), nitrilotriacetic acid (NTA), carboxymethylated aspartic acid (CM-Asp) and triscarboxymethyl ethylene diamine

(TED). By way of non-limiting example the solid support can be agarose beads, sepharose beads, acrylic beads, plastic microtiter plates, polyvinyltoluene (pvt) plastic (such as scintillation proximity assay (SPA) beads (Amersham (Piscataway, NJ) (Bosworth *et al.*, 1989, *Nature*, 341:167-168; Alouani, 2000, *Methods Mol. Biol.*, 138:135-41; Cook, 1996, *Drug Discov. Today*, 1: 287-294), magnetic beads, fluorescent beads, or polystyrene (such as FlashPlate®). SPA beads and FlashPlate® have solid scintillant embedded in the plastic, which permits the measurement of a bound radioactive label without rinsing or removal of any unbound labeled material. SPA beads are highly sensitive and easy to use in 96-well or larger format high throughput screening processed. FlashPlate® is a white polystyrene microplate designed for high-volume, in-plate radiobinding assays. The interior of each well is permanently coated with a thin layer of polystyrene-based scintillant that provides a platform for nonseparation assays using a variety of isotopes without the addition of liquid scintillation cocktail.

[0041] In some embodiments of the present invention, the metal ion is immobilized to SPA beads.

[0042] Labeled inositol phosphate can be detected by many methods known to those of skill in the art. Methods of detecting bound labeled inositol phosphate include, but are not limited to, radioactivity counting, light absorption, fluorescence or chemiluminescence measurement, and colorimetric measurement. The method of detection will depend on the type of label used and the type of solid support material. For example, if SPA beads or FlashPlate® are used as the solid support, radiolabeled inositol phosphate can be measured directly by using a scintillation counter (such as Topcount® NXT® or MicroBeta® Counter, Perkin-Elmer Life Sciences (Boston, MA). In other detection methods, unbound material may need to be removed prior to measurement of the bound labeled inositol phosphate. Methods of removal of unbound labeled material and retention of bound labeled material will be readily apparent to those of skill in the art, and include, but are not limited to, filtration or centrifugation.

[0043] In another aspect, the present invention is directed to methods for identifying compounds that modulate phospholipase C-linked receptor activation. These methods are carried out in the presence or absence of a test compound, and cells expressing a receptor that utilizes a phospholipase C signaling pathway are contacted with labeled inositol and with a receptor agonist, whereby labeled inositol phosphate is generated. The labeled inositol phosphate is released from the cells and contacted with an immobilized metal ion under conditions to allow inositol phosphate to bind to the metal ion. The bound labeled inositol phosphate is detected. An alteration in the amount of bound labeled inositol phosphate

detected when the assay is carried out in the presence of a test compound identifies the test compound as a compound that modulates phospholipase C-linked receptor activation.

[0044] Another aspect of the present invention relates to methods for detecting inositol monophosphatase activity in a sample comprising contacting the sample with labeled inositol phosphate under conditions permitting inositol monophosphatase to hydrolyze phosphate from inositol phosphate, contacting the sample with an immobilized metal ion under conditions permitting inositol phosphate to bind to the metal ion, and detecting labeled inositol phosphate bound to the immobilized metal ion. A decrease in the amount of bound labeled inositol phosphate, as compared with a control, is indicative of inositol monophosphatase activity in the sample.

[0045] Detection of the presence of functionally active inositol monophosphatase in a sample is based upon the measurement of inositol monophosphatase activity in the sample using metal ions to bind labeled inositol phosphate for quantification of inositol monophosphatase-catalyzed hydrolysis of input labeled inositol phosphate. A decrease in the amount of labeled inositol phosphate detected in the output, or as compared to a negative control, is indicative of the presence of inositol monophosphatase activity in the sample.

[0046] Any sample may be tested for the presence of inositol monophosphatase enzyme and its activity. Samples may come from any source including, but not limited to, biological sources. Examples of biological samples that can be assayed with the methods of the present invention include, but are not limited to, cerebrospinal fluid, serum or tissue extracts.

[0047] Labels for the inositol phosphate include, but are not limited to, radioactive, fluorescent, chemiluminescent, or hapten labels.

[0048] Conditions that allow inositol monophosphatase to catalyze the hydrolytic reaction to remove phosphate from the inositol phosphate are known to those of skill in the art and include, but are not limited to, a pH ranging from about 6.0 to about 8.0, and temperatures ranging from about 10°C to about 40°C.

[0049] In some embodiments of the present invention, the hydrolysis reaction is terminated prior to contacting the sample with the immobilized metal ion.

[0050] Methods of terminating the reaction are known to those of skill in the art and include, but are not limited to, adding an acidic solution to render the pH of the reaction mixture in a range of about 2.0 to about 4.0.

[0051] Another aspect of the present invention relates to methods for identifying compounds that modulate inositol monophosphatase activity comprising, in the presence or absence of a compound, contacting inositol monophosphatase with labeled inositol phosphate under

conditions permitting inositol monophosphatase to hydrolyze phosphate from inositol phosphate, contacting the reaction mixture with an immobilized metal ion under conditions permitting inositol phosphate to bind to the metal ion, and detecting labeled inositol phosphate bound to the immobilized metal ion, wherein an alteration in the amount of bound labeled inositol phosphate in the presence of a compound identifies said compound as a compound that modulates inositol monophosphatase activity.

[0052] Any form of functional inositol monophosphatase can be used in the methods of the present invention, including, but not limited to, purified native enzyme, recombinantly expressed enzyme, and naturally occurring or genetically-manipulated mutant or variant forms of the enzyme, in any state of purity.

[0053] In some embodiments of the present invention, purified inositol monophosphatase is used.

[0054] In some embodiments of the present invention the hydrolysis reaction is terminated prior to contacting the sample with the immobilized metal ion.

[0055] In another aspect of the present invention, methods are provided for detecting inositol-1-phosphate synthase activity in a sample comprising contacting the sample with labeled inositol under conditions permitting inositol-1-phosphate synthase to catalyze addition of phosphate to inositol, contacting the sample with an immobilized metal ion under conditions permitting inositol phosphate to bind to the metal ion, and detecting labeled inositol phosphate bound to the immobilized metal ion; wherein an increase in the amount of bound labeled inositol phosphate as compared with a control is indicative of inositol-1-phosphate synthase in the sample.

[0056] Detection of the presence of inositol-1-phosphate synthase in a sample is based upon the measurement of inositol-1-phosphate synthase activity in the sample using metal ions to bind labeled inositol phosphate for quantification of inositol-1-phosphate synthase-catalyzed phosphorylation of input labeled inositol. An increase in the amount of labeled inositol phosphate detected in the output, or as compared to a negative control, is indicative of the presence of inositol-1-phosphate synthase in the sample.

[0057] Any sample may be tested for the presence of inositol-1-phosphate synthase enzymatic activity. Samples may come from any source including, but not limited to, biological sources. Examples of biological samples that can be assayed with the methods of the present invention include, but are not limited to, cerebrospinal fluid, serum, and tissue extracts.



[0058] Labels for the inositol include, but are not limited to radioactive, fluorescent, chemiluminascent, or hapten labels.

[0059] Conditions that allow inositol-1-phosphate synthase to catalyze the reaction that adds a phosphate group to inositol to yield inositol phosphate are known to those of skill in the art and include, but are not limited to, a pH ranging from about 6.0 to about 8.0, and temperatures ranging from about 10°C to about 40°C.

[0060] In some embodiments of the present invention, the kinase reaction is terminated prior to contacting the sample with the immobilized metal ion.

[0061] Methods of terminating the kinase reaction are known to those of skill in the art and include, but are not limited to, adding an acidic solution to render the pH of the reaction mixture in a range of about 2.0 to about 4.0.

[0062] Another aspect of the present invention relates to methods for identifying compounds that modulate inositol-1-phosphate synthase activity comprising, in the presence or absence of a compound, contacting inositol-1-phosphate synthase with labeled inositol under conditions permitting inositol-1-phosphate synthase to catalyze addition of phosphate to inositol, contacting the reaction mixture with an immobilized metal ion under conditions permitting inositol phosphate to bind to the metal ion, and detecting labeled inositol phosphate bound to the immobilized metal ion, wherein an alteration in the amount of bound labeled inositol phosphate in the presence of a compound identifies said compound as a compound that modulates inositol-1-phosphate synthase activity.

[0063] Any form of functional inositol-1-phosphate synthase can be used in the methods of the present invention, including, but not limited to, purified native enzyme, recombinantly expressed enzyme, and naturally occurring or genetically-manipulated mutant or variant forms of the enzyme, in any state of purity.

[0064] In some embodiments of the present invention, purified inositol-1-phosphate synthase is used.

[0065] In some embodiments of the present invention, the kinase reaction is terminated prior to contacting the sample with the immobilized metal ion.

[0066] The invention is further illustrated by way of the following examples, which are intended to elaborate several embodiments of the invention. These examples are not intended to, nor are they to be construed to, limit the scope of the invention. It will be clear that the invention may be practiced otherwise than as particularly described herein. Numerous modifications and variations of the present invention are possible in view of the teachings herein and, therefore, are within the scope of the invention.

**EXAMPLES****Example 1. Materials and Methods.****Materials**

[0067] Myo- $^3\text{H}$ inositol (specific activity=110 Ci/mmol) and D-myo $^3\text{H}$ inositol-1-phosphate (specific activity=20 Ci/mmol) were purchased from Amersham Biosciences Corp. (Piscataway, NJ) and American Radiolabeled Chemicals, Inc. (St. Louis, MO), respectively. ATP, formic acid, acetic acid, MES, MOPS, HEPES, LiCl, FeCl<sub>3</sub>, CaCl<sub>2</sub>, MgCl<sub>2</sub>, NiCl<sub>2</sub>, CuCl<sub>2</sub>, substance P and L-733060 were from Sigma-Aldrich (St. Louis, MO). AlCl<sub>3</sub> was from Alfa Aesar (Ward Hill, MA). GaCl<sub>3</sub>, ScCl<sub>3</sub>, LuCl<sub>3</sub> and ZrOCl<sub>2</sub> were from Acros Organics (Morris Plains, NJ). Human recombinant platelet-derived growth factor (PDGF-BB) was from Calbiochem (San Diego, CA). Swiss 3T3 cell line was obtained from ATCC (Manassas, VA). The TopCount<sup>®</sup> NXT<sup>®</sup> Microplate Scintillation and Luminescence Counterinstrument was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA).

**Preparation of IMAC-SPA beads**

[0068] PVT SPA beads were custom coated with metal chelating compound, iminodiacetic acid (IDA), by Amersham Biosciences Corp. (Piscataway, NJ). Metal ions were loaded onto the beads by re-suspending 1 gram of beads in 40 ml solution of 100 mM AlCl<sub>3</sub>, FeCl<sub>3</sub>, GaCl<sub>3</sub>, ScCl<sub>3</sub>, LuCl<sub>3</sub>, or ZrOCl<sub>2</sub>. After 15 min of gentle rocking at room temperature, the free metal ions were removed by spinning down the beads and washing the beads 4 times with de-ionized water. The loaded SPA beads were re-suspended at 20 mg/ml in water or 20 mM formic acid.

**Characterization of inositol phosphate binding to IMAC-SPA beads**

[0069] To test whether IMAC-SPA beads could entrap inositol phosphate, but not inositol, 1 mg (100  $\mu\text{l}$ ) IMAC-SPA beads loaded with different metal ions were mixed with 10 nCi of  $^3\text{H}$ inositol (0.1pmol) or  $^3\text{H}$ inositol-1-phosphate (0.5pmol) in each well of a 96-well, 350ul Unifilter plate (Whatman). After 30 min of vigorous shaking, the binding mixtures were filtered using a Multiscreen vacuum manifold. The flow-through samples were collected in a 96-well white opaque plate and their radioactivity determined by liquid scintillation counting on TopCount<sup>®</sup> NXT<sup>®</sup> after adding 200  $\mu\text{l}$  of Microscint. The radioactivity trapped on the beads was measured directly by SPA on TopCount<sup>®</sup> NXT<sup>®</sup>.

[0070] To test the pH effect on the binding of inositol phosphate to IMAC-SPA beads, the beads were re-suspended in 20 mM different buffers, including formate (pH 3.0), acetate (pH 4.0 and pH 5.0), MES (pH 6.0), MOPS (pH 7.0) and HEPES (pH 8.0), mixed with 10 nCi of [ $^3\text{H}$ ]inositol-1-phosphate for 5 min and the bound radioactivity quantified using TopCount<sup>®</sup> NXT<sup>®</sup>.

[0071] The binding capacity of  $\text{Zr}^{4+}$ -SPA beads was determined by testing the ability of increasing concentrations of unlabeled myo-inositol-1-phosphate or ATP to block the binding of [ $^3\text{H}$ ]inositol-1-phosphate to the beads.

#### **Cell culture and receptor stimulation**

[0072] CHO cells stably expressing human neurokinin NK1 receptors were maintained in 5%  $\text{CO}_2$  and at 37°C in Ham's F12 medium supplemented with 10% fetal bovine serum (FBS) and 0.5mg/ml G418. For experiments, the cells were plated onto 96-well plates in inositol-free DMEM medium containing 10% FBS and 5  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]inositol (0.5  $\mu\text{Ci/well}$ ). After 16-48 hr incubation, the medium was removed and the cells were incubated for 15 min with or without NK1 antagonist in phosphate-free lithium-containing Hanks solution (PFLH) (composition: 20 mM HEPES, pH 7.4, 1.25 mM  $\text{MgCl}_2$ , 1.25 mM  $\text{CaCl}_2$ , 5 mM KCl, 125 mM NaCl, 10 mM LiCl and 10 mM glucose). The cells were then exposed to various concentrations of NK1 agonist, Substance P, for 45-60 min in PFLH solution at 37°C. At the end of the incubation, the agonist solution was removed and 100  $\mu\text{l}$  ice-cold 20 mM formic acid solution (pH3.0) containing 2 mM myo-inositol was added in each well to release inositol phosphates from the cells. After incubation at 4°C for over 10 min, the samples were transferred to a white opaque plate, and 100  $\mu\text{l}$  of metal ion loaded IMAC-SPA beads added to each well. Alternatively, IMAC-SPA beads could be added directly to the cell plate to eliminate the sample transfer step. The amount of [ $^3\text{H}$ ] inositol phosphates generated in the cells was then determined by measurement of the radioactivity on the SPA beads on TopCount<sup>®</sup> NXT<sup>®</sup>.

[0073] Swiss 3T3 cells were maintained in DMEM medium supplemented with 10% heat-inactivated calf serum. For PI hydrolysis assay, the cells were grown in 96-well plates in [ $^3\text{H}$ ]inositol-containing medium for 16-48 hr, then deprived of serum for 24 hr. The cells were stimulated with PDGF-BB for 45-60 min and the inositol phosphate accumulated in the cells was measured by IMAC-SPA in the same way as described for NK1 receptors.

**Example 2. Measurement of [ $^3\text{H}$ ]Inositol Phosphate Generated From PI Hydrolysis.**

[0074] Metal ions, immobilized on a solid support as an affinity matrix, were used to bind and isolate radiolabeled inositol phosphate, which was subsequently quantified by SPA technology. SPA beads are microspheres 5 microns in diameter consisting of a solid scintillant-containing polyvinyltoluene core coated with a polyhydroxy film (Cook, *supra*). A metal chelating compound, iminodiacetic acid, was covalently attached to the coating, allowing metal ions to be immobilized on the SPA beads. A phosphate moiety interacts with an immobilized metal ion through two coordination bonds and forms a strong four-member ring complex (Andersson *et al.*, *supra*; Chaga *et al.*, *supra*; Muszynska *et al.*, *supra*; Holmes *et al.*, *supra*). The binding of [ $^3\text{H}$ ]inositol phosphates to the SPA beads via the interaction of their phosphate moieties with the immobilized metal ions brought the radioisotope in close proximity to the scintillant embedded in the beads and caused energy transfer and photon emission which were readily detected by TopCount<sup>®</sup> NXT<sup>®</sup> or MicroBeta<sup>®</sup> Reader (PerkinElmer Life Science).

**Example 3. Interactions of Inositol Phosphate With IMAC-SPA Beads.**

A number of metal ions can be immobilized on solid support via IDA groups (Sulkowski, *supra*; Yip *et al.*, *supra*; Chaga, 2001, J. Biochem. Biophys. Methods, 49:313-334). Metal ions can be divided into three categories (hard, intermediate and soft) based on their preferential reactivity towards nucleophiles (Chaga, 2001, *supra*; Pearson, R., 1973, Hard and Soft Acids and Bases, pp 53 – 85, Hutchinson & Ross, Stroudsburg, PA). The hard Lewis metal ions ( $\text{Al}^{3+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Lu}^{3+}$ ,  $\text{Sc}^{3+}$ ,  $\text{Zr}^{4+}$ ) show preference for oxygen, while the soft metal ions ( $\text{Cu}^+$ ,  $\text{Hg}^{2+}$ ,  $\text{Ag}^+$ ) prefer sulfur. The intermediate (or transition) metal ions ( $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Co}^{2+}$ ) coordinate nitrogen, oxygen and sulfur. To identify the metal ions which could be used as an affinity ligand to selectively entrap inositol phosphate onto the SPA beads, we immobilized 10 different metal ions onto SPA beads, incubated the beads with [ $^3\text{H}$ ]inositol-1-phosphate or [ $^3\text{H}$ ]inositol and then used vacuum filtration to separate the bound from free inositides. As shown in Figure 1, there was no significant binding of [ $^3\text{H}$ ]inositol to SPA beads loaded with any of the metal ions. The amount of inositol radioactivity retained on the beads was at background level and was independent of the metal ions used. In contrast to inositol, the amount of free [ $^3\text{H}$ ]inositol-1-phosphate in the flow-through samples was reduced by various degrees depending on the metal ions that were immobilized on the beads. The loss of radioactivity in the flow-through corresponds to the retention on the SPA beads. Immobilized hard metal ions  $\text{Al}^{3+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Ga}^{3+}$ ,  $\text{Lu}^{3+}$ ,  $\text{Sc}^{3+}$  and  $\text{Zr}^{4+}$  adsorbed 80-90% and  $\text{Ca}^{2+}$

adsorbed 20% of [ $^3\text{H}$ ]inositol-1-phosphate, while the transition metal ions  $\text{Cu}^{2+}$  and  $\text{Ni}^{2+}$  did not adsorb a significant amount. The radioactivity of [ $^3\text{H}$ ]inositol-1-phosphate trapped on the beads was measured by SPA technology which had a ~50% lower efficiency than liquid scintillation counting, therefore the total cpm in the SPA samples underestimated the amount of radioactivity that was adsorbed. Among the metal ions used, only  $\text{Ni}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Fe}^{3+}$  have colors. Due to the color quenching effect, the radioactivity on the  $\text{Fe}^{3+}$ -loaded SPA beads was much lower than that on the  $\text{Zr}^{4+}$ -loaded beads, although the amount of [ $^3\text{H}$ ]inositol-1-phosphate retained on the beads were very similar. These data indicate that several hard metal ions, including  $\text{Zr}^{4+}$ ,  $\text{Al}^{3+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Ga}^{3+}$ ,  $\text{Lu}^{3+}$  and  $\text{Sc}^{3+}$ , can be immobilized on the SPA beads and utilized as affinity ligands to entrap and quantify [ $^3\text{H}$ ]inositol phosphates in the solution.

**Example 4. Effect of pH on Inositol Phosphate Binding to IMAC-SPA Beads.**

[0075] To evaluate the effect of pH on the assay, the binding of [ $^3\text{H}$ ]inositol-1-phosphate to  $\text{Zr}^{4+}$ -SPA beads was carried out in solutions containing 20 mM buffer at different pH ranging from 3.0 to 8.0. As shown in Figure 2, at pH 6.0 or below, the binding was optimal. At pH 7.0, the binding was significantly reduced, and at pH 8.0, the binding was almost completely abolished. Since the binding of metal ions to IDA and the SPA counting efficiency are not affected by neutral pH (Cook, 1996, *supra*; Chaga, 2001, *supra*; Porath 1992, Protein Expr. Purif., 3:263-281), it is likely that the protonation status of the phosphate group, which has a pKa at ~7.2, affects the binding of [ $^3\text{H}$ ]inositol-1-phosphate to the immobilized metal ions. Use 20 mM formic acid (pH3.0) effectively released inositol phosphates from the cells after stimulation for measurement of PI hydrolysis by IMAC-SPA.

**Example 5. Assessment of Binding Capacity of  $\text{Zr}^{4+}$ -SPA beads.**

[0076] To determine the binding capacity of  $\text{Zr}^{4+}$ -SPA beads, the binding of 10 nCi [ $^3\text{H}$ ]inositol-1-phosphate to 2 mg of the beads, pre-incubated with increasing concentrations of unlabeled inositol phosphate or ATP, was measured. As shown in Figure 3, pre-treatment of 2 mg beads with up to 1 nmol unlabeled inositol phosphate or ATP did not block the binding of [ $^3\text{H}$ ]inositol-1-phosphate to  $\text{Zr}^{4+}$ -SPA beads, indicating the binding capacity is at ~0.5 nmol/mg beads.

**Example 6. Measurement of NK1-Mediated Response.**

[0077] The neurokinin NK1 receptor is a member of the seven-transmembrane-domain G protein coupled receptor superfamily. Through the coupling of Gq/11 class of heterotrimeric G protein, stimulation of NK1 receptor by agonists, such as Substance P, triggers the activation of PLC- $\beta$  and results in an increase in PI hydrolysis (Severini *et al.*, 2002, Pharmacol. Rev., 54:285-322; Seabrooka *et al.*, 1996, Eur. J. Pharmacol., 317:129-135).

[0078] To test whether metal ion-loaded SPA beads can be used to measure NK1-mediated PI hydrolysis, CHO cells, stably expressing NK1 receptor, were incubated with [ $^3$ H]inositol and stimulated with Substance P in the presence of 10 mM LiCl. The acid-soluble components of the cells were then released into 20 mM formic acid solution and mixed with SPA beads, loaded with Hard Lewis metal ions, to measure the [ $^3$ H]inositol phosphates generated from PI hydrolysis. Figure 4 depicts the radioactivity released from cells treated with saline (control) or 0.1  $\mu$ M Substance P as detected by the SPA beads with immobilized  $\text{Al}^{3+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Ga}^{3+}$ ,  $\text{Lu}^{3+}$ ,  $\text{Sc}^{3+}$  and  $\text{Zr}^{4+}$ . Significant increases in [ $^3$ H]inositol phosphate production in the cells stimulated with Substance P were detected by all six immobilized hard Lewis metal ions. The  $\text{Zr}^{4+}$ -loaded SPA beads gave the best performance, yielding the highest cpm and a 12-fold increase in signal over the control.

**Example 7. Optimization of Amount of SPA Beads.**

[0079] To optimize the assay conditions, different amounts of SPA beads were added to the wells of a 96-well plate to measure Substance P-stimulated PI hydrolysis. As shown in Figure 5, the best results can be achieved by using 1 mg/well or 2 mg/well  $\text{Zr}^{4+}$ -loaded SPA beads. The lower cpm with 4 mg/well bead was probably due to the stacking effect of the beads that blocked the light path. To further simplify the assay procedures, the cells were plated on white opaque 96-well plates, and  $\text{Zr}^{4+}$ -SPA beads in 20 mM formic acid (pH3.0) were added directly to the cell plate after receptor stimulation. However, elimination of the transfer step resulted in only a 2-fold increase in background and 2-fold reduction of the assay window.

**Example 8. Evaluation of Functional Properties of NK1 Receptor Agonist and Antagonist Using  $\text{Zr}^{4+}$ -SPA Beads.**

[0080] To ensure that the measurement of PI hydrolysis by this new approach does not change the receptor pharmacology, we evaluated the functional properties of a NK1 receptor

agonist and antagonist using  $\text{Zr}^{4+}$ -SPA beads. Figure 6 depicts the concentration-response curves of Substance P-induced PI hydrolysis with or without the pre-treatment of the cells with NK1 antagonist L-733060. Substance P stimulated PI hydrolysis in NK1/CHO cells in a concentration-dependent manner with an  $\text{EC}_{50}$  value of 0.15 nM. L-733060 inhibited competitively the PI hydrolysis induced by Substance P and shifted the concentration-response curve to the right. From Schild regression analysis, the functional potency ( $\text{pK}_a$ ) of the antagonist was determined to be close to 9.0, which is consistent to the values reported in the literature (Seabrooka *et al.*, *supra*; Noailles *et al.*, 2002, Ann. NY Acad. Sci., 965:267-73; Rupniak *et al.*, 2000, Neuropharmacology, 39:1413-1421).

**Example 9. Measurement of PDGF Receptor-Mediated Response.**

[0081] The PDGF receptor belongs to the superfamily of tyrosine kinase-linked growth factor receptors. Stimulation of the PDGF receptor leads to activation of PLC- $\gamma$  through Ras-related GTPase (Ji *et al.*, 1999, Mol. Cell. Biol., 19:4961-4970; Wang *et al.*, 1998, Mol. Cell. Biol., 18:590-597; Heldin *et al.*, 1998, Biochim. Biophys. Acta, 1378:F79-113; Stice *et al.*, 1999, Front. Biosci., 4:D72-86). To demonstrate the use of immobilized metal ions on SPA beads for the measurement of PI hydrolysis mediated by growth factor receptors, quiescent Swiss 3T3 cells were stimulated with PDGF-BB, and the amount of [ $^3\text{H}$ ]inositol phosphates generated in the cells was determined using  $\text{Zr}^{4+}$ -SPA beads. As shown in Figure 7, PDGF stimulated PI hydrolysis in a concentration-dependent manner with a maximal 5-fold increase in inositol phosphate production and an  $\text{EC}_{50}$  value of 3.0 ng/ml. The  $\text{EC}_{50}$  value determined by this new approach was not significantly different from the values reported in the literature (Berridge *et al.*, 1984, *supra*; Blakeley *et al.*, 1989, Biochem. J., 258:177-85; Chu *et al.*, 1985, J. Cell. Physiol., 124:391-396).

[0082] The foregoing examples are meant to illustrate the invention and are not to be construed to limit the invention in any way. Those skilled in the art will recognize modifications that are within the spirit and scope of the invention.

**We claim:**

1. A method for detecting phospholipase C-linked receptor activation comprising
  - a) providing cells expressing a receptor that utilizes a phospholipase C signaling pathway;
  - b) contacting the cells with labeled inositol;
  - c) contacting the cells with a receptor agonist, whereby labeled inositol phosphate is generated;
  - d) releasing the labeled inositol phosphate from the cells;
  - e) contacting the cells with an immobilized metal ion under conditions permitting inositol phosphate to bind to the metal ion; and
  - f) detecting labeled inositol phosphate bound to the immobilized metal ion;wherein bound labeled inositol phosphate is indicative of receptor activation.
2. The method of claim 1, wherein the receptor is a seven transmembrane domain G protein-coupled receptor or a single transmembrane domain tyrosine kinase-linked receptor.
3. The method of claim 2, wherein the receptor is selected from neurokinin NK1 receptor, muscarinic m1 acetylcholine receptor, PDGF receptor, and NGF receptor.
4. The method of claim 1, wherein the metal ion is selected from  $\text{Zr}^{4+}$ ,  $\text{Ga}^{3+}$ ,  $\text{Al}^{3+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Sc}^{3+}$ , and  $\text{Lu}^{3+}$ .
5. The method of claim 5, wherein the metal ion is  $\text{Zr}^{4+}$ .
6. The method of claim 1, wherein the metal ion is immobilized to SPA beads.
7. The method of claim 1, wherein the inositol phosphate label is selected from radiolabel, fluorescent, chemiluminescent, and hapten.
8. The method of claim 8, wherein the inositol phosphate label is a radiolabel.
9. A method for identifying compounds that modulate phospholipase C-linked receptor activation comprising, in the presence or absence of a compound,



- a) providing cells expressing a receptor that utilizes a phospholipase C signaling pathway;
- b) contacting the cells with labeled inositol;
- c) contacting the cells with a receptor agonist, whereby labeled inositol phosphate is generated;
- d) releasing labeled inositol phosphate from the cells;
- e) contacting the labeled inositol phosphate with an immobilized metal ion under conditions to allow inositol phosphate to bind to the metal ion; and
- f) detecting labeled inositol phosphate bound to the immobilized metal ion;

wherein an alteration in the amount of bound labeled inositol phosphate in the presence of a compound identifies said compound as a compound that modulates phospholipase C-linked receptor activation.

10. A method for detecting inositol monophosphatase activity in a sample comprising

- a) contacting the sample with labeled inositol phosphate under conditions permitting inositol monophosphatase to hydrolyze phosphate from inositol phosphate;
- b) contacting the sample with an immobilized metal ion under conditions permitting inositol phosphate to bind to the metal ion; and
- c) detecting labeled inositol phosphate bound to the immobilized metal ion;

wherein a decrease in the amount of bound labeled inositol phosphate is indicative of inositol monophosphatase activity in the sample.

11. The method of claim 10, wherein the hydrolysis reaction is terminated prior to contacting the sample with the immobilized metal ion.

12. A method for identifying compounds that modulate inositol monophosphatase activity comprising, in the presence or absence of a compound,

- a) contacting inositol monophosphatase with labeled inositol phosphate under conditions permitting inositol monophosphatase to hydrolyze phosphate from inositol phosphate;
- b) contacting the reaction mixture of step a) with an immobilized metal ion under conditions permitting inositol phosphate to bind to the metal ion; and
- c) detecting labeled inositol phosphate bound to the immobilized metal ion;

wherein an alteration in the amount of bound labeled inositol phosphate in the presence of a compound identifies said compound as a compound that modulates inositol monophosphatase activity.

13. The method of claim 12, wherein the hydrolysis reaction is terminated prior to contacting the sample with the immobilized metal ion.

14. A method for detecting inositol-1-phosphate synthase activity in a sample comprising

- a) contacting the sample with labeled inositol under conditions permitting inositol-1-phosphate synthase to catalyze addition of phosphate to inositol;
- b) contacting the sample with an immobilized metal ion under conditions permitting inositol phosphate to bind to the metal ion; and
- c) detecting labeled inositol phosphate bound to the immobilized metal ion;

wherein an increase in the amount of bound labeled inositol phosphate is indicative of inositol-1-phosphate synthase activity in the sample.

15. The method of claim 14, wherein the kinase reaction is terminated prior to contacting the sample with the immobilized metal ion.

16. A method for identifying compounds that modulate inositol-1-phosphate synthase activity comprising, in the presence or absence of a compound,

- a) contacting inositol-1-phosphate synthase with labeled inositol under conditions permitting inositol-1-phosphate synthase to catalyze addition of phosphate to inositol;
- b) contacting the reaction mixture of step a) with an immobilized metal ion under conditions permitting inositol phosphate to bind to the metal ion; and
- c) detecting labeled inositol phosphate bound to the immobilized metal ion;

wherein an alteration in the amount of bound labeled inositol phosphate in the presence of a compound identifies said compound as a compound that modulates inositol-1-phosphate synthase activity.

17. The method of claim 16, wherein the kinase reaction is terminated prior to contacting the sample with the immobilized metal ion.

**ABSTRACT**

Assays for detecting inositol phosphate, for measuring the activity of phospholipase C-linked receptors, inositol kinases, and inositol phosphatases, and for screening for compounds that modulate the activity of these receptors and enzymes.

FIG. 1A

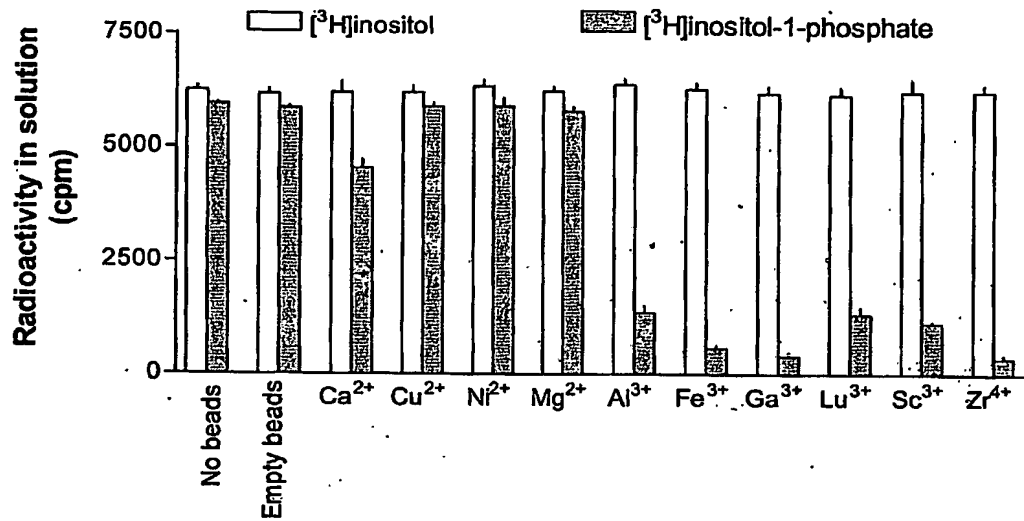


FIG. 1B

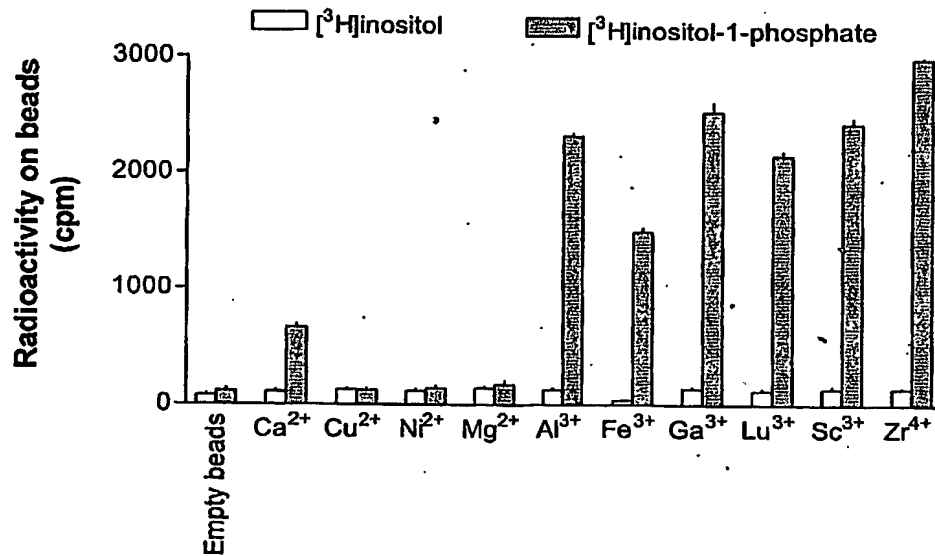


FIG. 2

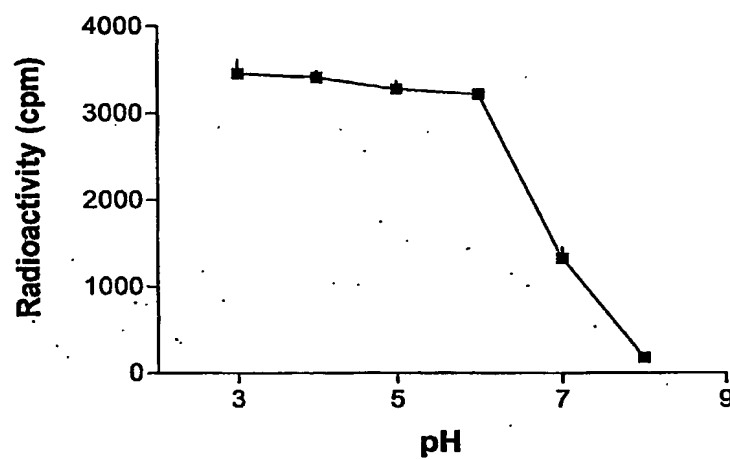


FIG. 3

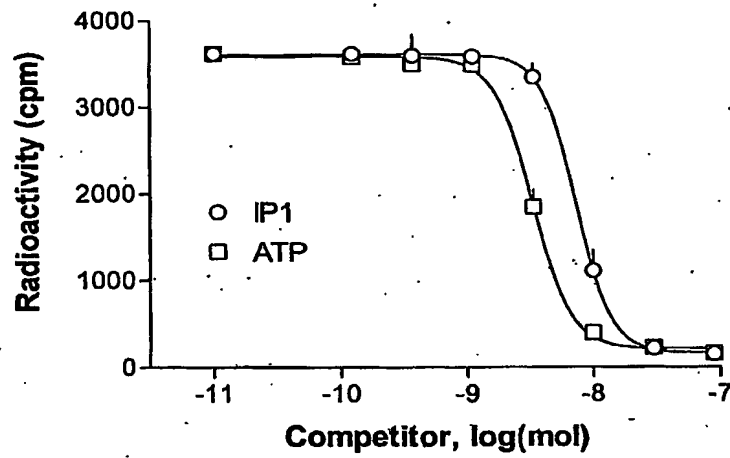


FIG. 4

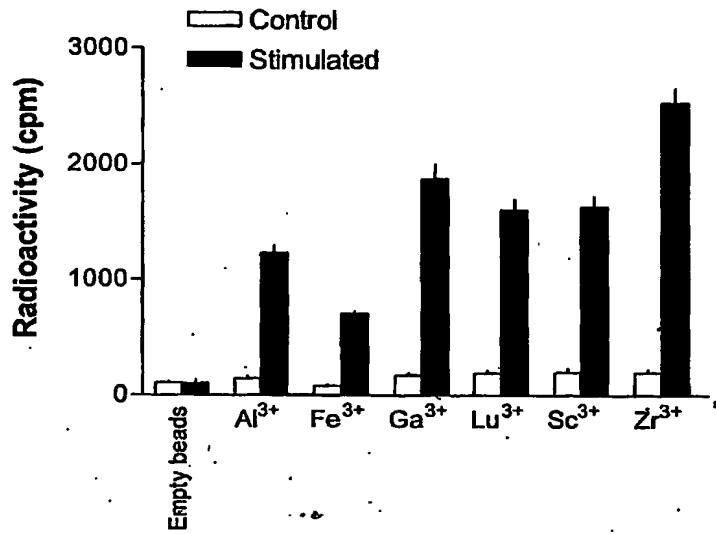


FIG. 5

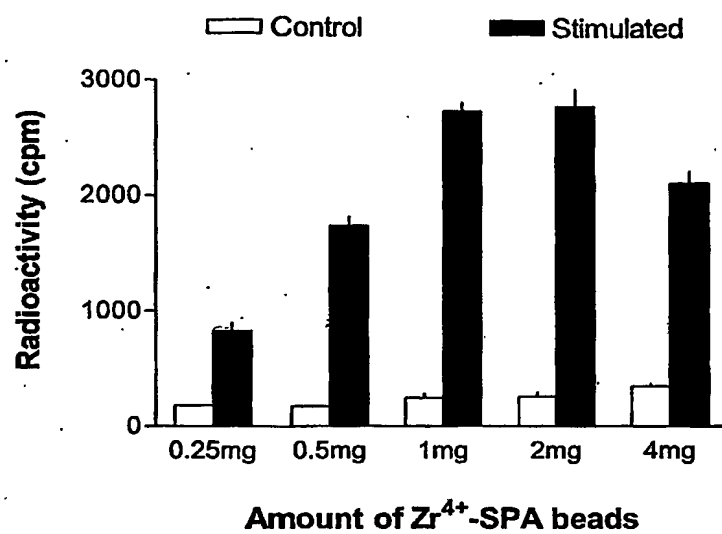




FIG. 6

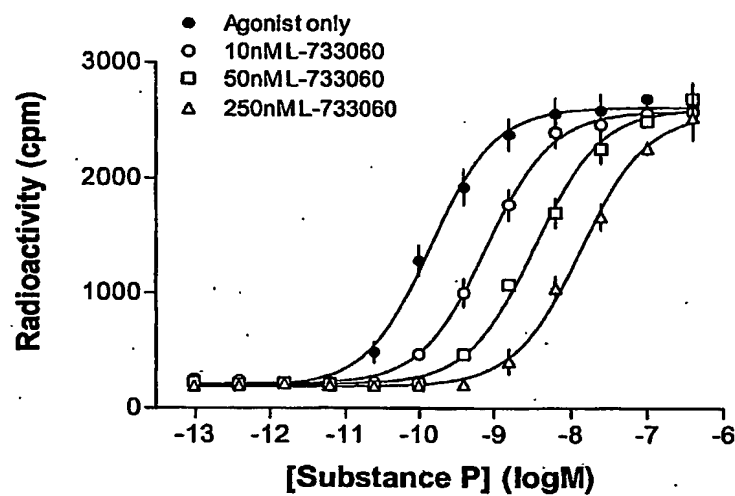
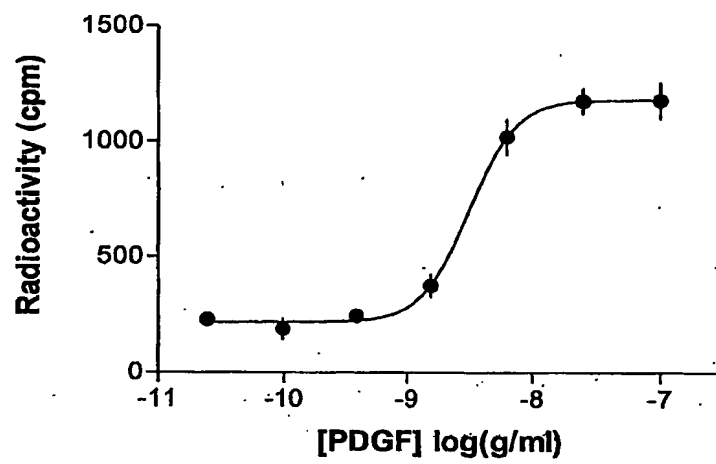


FIG. 7



This Page is inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

## BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ BLACK BORDERS
- ☒ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☒ COLORED OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REPERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images problems checked, please do not report the problems to the IFW Image Problem Mailbox**